

(19)



Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

EP 0 835 931 A1

(12)

EUROPEAN PATENT APPLICATION

published in accordance with Art. 158(3) EPC

(43) Date of publication:

15.04.1998 Bulletin 1998/16

(51) Int. Cl.⁶: **C12N 9/96**, C12Q 1/37,

C12Q 1/56

(21) Application number: **96918887.9**

(86) International application number:

PCT/JP96/01738

(22) Date of filing: **24.06.1996**

(87) International publication number:

WO 97/01631 (16.01.1997 Gazette 1997/04)

(84) Designated Contracting States:

DE FR

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(30) Priority: **26.06.1995 JP 159330/95**

26.06.1995 JP 159331/95

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(54) **STABLE PLASMIN SOLUTION**

(57) The present invention relates to a plasmin solution comprising:

(A) plasmin and

(B) the following component (B-1), (B-2) or (B-3):

(B-1) an oligopeptide comprising at least two amino acids bonded each other, said two amino acids being selected from lysine, arginine, glycine, alanine, aspartic acid and methionine;

(B-2) at least two amino acids selected from lysine, arginine, glycine, alanine, aspartic acid and methionine; or

(B-3) an amino acid selected from lysine, arginine, glycine, alanine, aspartic acid and methionine and a polyhydric alcohol. This solution is maintained stably without deteriorations in the plasmin activity and binding activity with α 2PI even after long-term storage so that it is useful as a reagent for the quantitative measurement of α 2PI.

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Description**Technical Field**

5 The present invention relates to a stable plasmin solution useful as a reagent for the quantitative measurement of a blood coagulation and fibrinolysis factor.

Background Art

10 The reaction of an enzyme in the living body is controlled and regulated by its activating substance or a substance inhibiting its reaction, whereby its function is adjusted. For example, in the blood coagulation mechanism, there exists an enzyme inhibitor which inhibits the blood coagulation reaction at the sites other than a vascular injury site or inhibits hypercoagulability and hyperfibrinolysis and by the inhibitor, blood coagulation and fibrinolysis is being controlled and regulated. In the hematological test to investigate the advance of thrombus formation, the quantitative measurement of
 15 an enzyme inhibitor has an important meaning. Since the amount of the enzyme inhibitor serves as a suitable index showing the condition of coagulation and fibrinolysis, quantitative measurement of an enzyme inhibitor such as anti-thrombin III (ATIII) or α 2-plasmin inhibitor (α 2PI) is carried out.

It has been made cleared that among such enzyme inhibitors, α 2PI is one of the most important fibrinolysis inhibiting factors which can control fibrinolysis in the blood and it has attracted attentions as an index for finding hyperfibrinolysis in the living body. The blood level of α 2PI is different according to the kinds or symptoms of the diseases. For
 20 example, it shows a marked reduction in the case of disseminated intravascular coagulation (DIC) or liver troubles. The blood level of α 2PI has therefore been used as an index for screening of such a disease, analysis of the morbid state or judgment of prognosis and also an index for judging drug efficacy at the fibrinolytic treatment.

The quantitative measurement of such an enzyme inhibitor has conventionally been carried out by reacting the
 25 enzyme inhibitor with an excess amount of an enzyme and then measuring the residual amount of the enzyme. For example, based on the fact that α 2PI inhibits the enzyme plasmin, the amount of α 2PI in a biosample (specimen) is measured by reacting α 2PI in the specimen with a certain amount of plasmin and then measuring the remaining plasmin activity. In this case, the activity of the plasmin is determined, for example, by measuring the hydrolysis rate of a chromogenic synthetic substrate from a change in the absorbance.

30 Many of such enzyme inhibitors are serine protease inhibitors and an enzyme for measuring such enzyme inhibitors is serine protease. Proteases such as serine protease each has a site which will become its own substrate within its molecule so that prompt decomposition in the solution and lowering in the protease activity or binding activity with a protease inhibitor are sometimes observed. For example, a human-derived plasmin used for the quantitative measurement of α 2PI loses 72% of its protease activity when allowed to stand at 37°C for one hour and decomposition occurs
 35 both in the H chain and L chain (K.N.N. Reddy, Biochem. Biophys. Res. Commun., **92**, 1016-1022(1980)).

It is known, on the other hand, that the protease activity of plasmin exhibits improved stability under the presence of fibrinogen, ϵ -aminocaproic acid and glycerol or at high ion strength (J. Jespersen, Thromb. Res., **37**, 3955-404(1986) or under the presence of ϵ -aminocaproic acid and lysine (K.N.N. Reddy, Progress in Fibrinolysis, 374-379(1981)).

40 The above methods are however accompanied with the drawbacks that they bring about stabilization only for extremely short time and if plasmin is allowed to stand at 37°C for one hour, its activity lowers considerably; and that even if 50% glycerol permitting the relative retention of the stability of protease activity is added, the binding activity with a protease inhibitor lowers (M. Shimokawa, Analytical Science, 10, 533-536 (1994)).

The reagent for the quantitative measurement of such an enzyme inhibitor is prepared as a lyophilized product because plasmin cannot be stored in the form of a solution. It must be prepared as a solution right before the measure-
 45 ment. Thus, the conventional reagent involves problems in economy, operability and prompt measurement.

In addition, it is difficult to employ an automatic analyzer for the measurement adopting the conventionally used plasmin solution as a measuring reagent of α 2PI, because the solution is highly viscous and besides, in the form of the solution, marked reductions in the activity of plasmin and binding activity of plasmin with α 2PI occur.

50 An object of the present invention is therefore to provide a process for stabilizing plasmin by which the plasmin activity and binding activity of plasmin with α 2PI can be maintained stably for a long time even after storage in the form of a solution; and a stable plasmin solution.

Brief Description of the Drawings

55 FIG. 1 illustrates a relationship between an α 2PI concentration and measured value (Δ Abs/min) when a calibration curve exactness was studied in Example 5.

Disclosure of the Invention

With the forgoing in view, the present inventors have carried out an extensive investigation. As a result, it has been found that a plasmin solution which is stable in plasmin activity and binding activity with α 2PI for a long time and is therefore useful as a reagent for the quantitative measurement of α 2PI can be obtained by using specific amino acids or an oligopeptide having said specific amino acids bonded each other, leading to the completion of the present invention.

The present invention therefore provides a plasmin solution comprising:

- (A) plasmin and
(B) the following component (B-1), (B-2) or (B-3):

(B-1) an oligopeptide comprising at least two amino acids bonded each other, said two amino acids being selected from lysine, arginine, glycine, alanine, aspartic acid and methionine;

(B-2) at least two amino acids selected from lysine, arginine, glycine, alanine, aspartic acid and methionine; or

(B-3) an amino acid selected from lysine, arginine, glycine, alanine, aspartic acid and methionine and a polyhydric alcohol.

The present invention also provides a process for the stabilization of plasmin, which comprises adding, to a plasmin solution,

(B-1) an oligopeptide comprising at least two amino acids bonded each other, said two amino acids being selected from lysine, arginine, glycine, alanine, aspartic acid and methionine;

(B-2) at least two amino acids selected from lysine, arginine, glycine, alanine, aspartic acid and methionine; or

(B-3) an amino acid selected from lysine, arginine, glycine, alanine, aspartic acid and methionine and a polyhydric alcohol.

Best Modes for Carrying Out the Invention

As plasmin usable in the present invention, any one of methionyl type (having methionine at its N terminal), glutamyl type (having glutamic acid at its N terminal) and lysyl type (having lysine at its N terminal) can be used. For example, plasmin commercially available from Chromogenics Inc. can be used. These types of plasmin can be used either singly or in combination and it is preferred to use it so that its activity falls within a range of 0.1 to 10 nkat/ml, particularly 0.3 to 5 nkat/ml. Incidentally, the term "1 nkat" as used herein means an amount of plasmin sufficient for decomposing 1 nmol of a plasmin synthetic substrate (S-2251) per second.

Examples of the oligopeptide usable as the component (B-1) in the present invention include dipeptides and tripeptides obtained by using, in combination, one or more amino acid selected from lysine, arginine, glycine, alanine, aspartic acid and methionine. Among them, dipeptides and tripeptides composed of one amino acid are preferred, with glycyl glycine, glycyl glycyl glycine and alanyl alanine being particularly preferred.

These oligopeptides can be used either singly or in combination. The oligopeptide is preferably added in an amount of 1 to 20 wt.% (which will hereinafter be indicated by "%", simply), particularly 5 to 20% based on the whole composition. Based on plasmin in the solution, it is preferably added in an amount ranging from 1 to 2000 mg/nkat, with a range of 10 to 700 mg/nkat being particularly preferred.

When at least two amino acids are used in combination as the component (B-2) in the present invention, they are selected from lysine, arginine, glycine, alanine, aspartic acid and methionine. Among them, the combination of lysine and glycine and that of lysine and alanine are preferred.

It is preferred that these amino acids are added in a total amount of 1 to 20 %, particularly 5 to 20% based on the whole composition. It is, on the other hand, preferred to add them in an amount ranging from 1 to 2000 mg/nkat, particularly 10 to 700 mg/nkat, relative to plasmin in the solution.

In the present invention, one of amino acids selected from lysine, arginine, glycine, alanine, aspartic acid and methionine and a polyhydric alcohol are used as the component (B-3).

As the amino acid usable here, lysine is particularly preferred.

The amino acid is preferably added in an amount of 1 to 20%, particularly 5 to 20% based on the whole composition. Relative to plasmin in the solution, on the other hand, the amino acid is preferably added in an amount ranging from 1 to 2000 mg/nkat, particularly 10 to 700 mg/nkat.

Preferred examples of the polyhydric alcohol usable here include glycerol, ethylene glycol and polyethylene glycol. The polyhydric alcohol is preferably added in an amount of 1 to 50%, particularly 5 to 30%, based on the whole composition.

When an oligopeptide (B-1) or at least two amino acids (B-2) are used as the component (B), a polyhydric alcohol can be added further to obtain a more stable plasmin solution. Examples of the polyhydric alcohol usable here include glycerol, ethylene glycol and polyethylene glycol. In the case where a polyhydric alcohol is added, it is preferably added in an amount of 1 to 50%, particularly 5 to 30% based on the whole composition.

5 When an oligopeptide (B-1) is used as the component (B), one or more amino acids selected from lysine, arginine, glycine, alanine, aspartic acid and methionine can be used in combination to obtain a more stable plasmin solution. In this case, these amino acids are preferably added in an amount of 1 to 20 %, particularly 5 to 20%, based on the whole composition.

10 When an oligopeptide (B-1) and one or more amino acids are used as the component (B), a polyhydric alcohol can be added further to obtain a more stable plasmin solution. As the polyhydric alcohol usable here, glycerol, ethylene glycol, polyethylene glycol and the like are preferred. When the polyhydric alcohol is added, it is preferably added in an amount of 1 to 50%, with 5 to 30% being particularly preferred.

15 The plasmin solution according to the present invention is useful as a reagent for the quantitative measurement of α 2PI, a standardized solution of plasmin or the like. When the plasmin solution is used as a reagent, quantitative determination of α 2PI in a specimen can be effected with high precision by using an ordinarily-employed chromogenic synthetic substrate. No particular limitation is imposed on the chromogenic synthetic substrate. For example, S-2251 (H-D-Val-Leu-Lys-paranitroaniline) or S-2403 (Glu-Phe-Lys-paranitroaniline) produced by Chromogenic Inc. can be suitably used.

20 **Examples**

The present invention will hereinafter be described more specifically by examples. It should however be borne in mind that the present invention is not limited to or by the following examples.

25 Example 1

To 250 μ l of a first reagent having the below-described composition, a normal plasma specimen (containing a pre-determined amount of α 2PI) and physiological saline were added, each in an amount of 3 μ l, respectively, followed by the reaction at 37°C for 5 minutes. To each of the reaction mixtures, 100 μ l of a second reagent having the below-described composition were added. A change in an absorbance at a wavelength of 405 nm per minute was measured. The change in an absorbance at the time when physiological saline was used indicates plasmin activity, that is, a titer to hydrolyze a plasmin synthetic substrate (S-2251) of plasmin. A value obtained by subtracting the remaining activity of plasmin after the reaction of the normal plasma specimen from the plasmin activity of physiological saline indicates the binding activity of plasmin with α 2PI.

35 As a solution to be used for the plasmin solution of a second reagent, used was a 20% glycerol solution containing an additive which will be shown in Table 1. The solution was adjusted to pH 7.4 with sodium hydroxide or hydrochloric acid and then plasmin was dissolved in it. A half portion of the solution was used for measurement on that day, while the remaining half portion was provided for measurement after hermetically sealed and allowed to stand at 37°C for 4 days. Incidentally, 50% glycerol and 20% glycerol were used for comparison. The results are shown in Table 1.

40

First reagent:	
H-D-Valyl-L-leucyl-L-lysyl-p-nitroaniline ("S-2251" produced by Chromogenic Inc.)	1.2 mM
25 mM tris buffer (pH 7.4)	

45

50

Second reagent:	
Plasmin	3 nKat/ml
Each plasmin solution (pH 7.4)	
*: the term "1 nkat" means an amount of plasmin sufficient for decomposing 1 nmol of S-2251 per second.	

55

Table 1

No.	Additive	Initial value		Measured after 4 days at 37 °C		Ratio (%)	
		Activity	Binding activity	Activity	Binding activity	Activity	Binding activity
1	50% glycerol	3517	646	3520	514	100	80
2	20% glycerol	2722	620	293	83	3	3
3	10% lysine	3017	416	2960	377	98	91
4	10% arginine	2916	570	2678	388	92	68
5	10% alanine	2969	660	1974	501	66	76
6	10% glycine	2853	604	1945	457	68	76
7	10% glycly glycine	2668	416	2655	352	100	85

(1) Additives in Nos. 3 to 7 were dissolved in a 20% glycerol solution.

(2) The initial values and values measured when the plasmin solution was allowed to stand at 37°C for 4 days are each indicated by the unit of Δ Abs/min.

(3) "Activity" means a titer to hydrolyze a plasmin synthetic substrate (S-2251).

(4) "Binding activity" means the binding ability of plasmin with α 2PI and is a value obtained by subtracting, from the activity value of plasmin, residual plasmin activity at the time when the normal plasma specimen was reacted.

(5) "Ratio" means a ratio of the activity or binding activity measured after the plasmin solution was allowed to stand at 37°C for 4 days supposing that the activity or binding activity at the initial stage is 100%, respectively.

As is apparent from the results in Table 1, it has been found that the plasmin solution according to the present invention was maintained stably without deteriorations in the plasmin activity and α 2PI binding activity and that the viscosity of the plasmin solution was low.

Example 2

As in Example 1, various plasmin solutions having the compositions as Shown in Table 2 were prepared and the plasmin activity and binding activity of plasmin with α 2PI were measured at the time when the solutions were prepared and after the solutions were stored at 37°C for 2 days. The results are shown in Table 2.

Table 2

No.	Additive	Initial value		Measured after 2 days at 37 °C		Ratio (%)	
		Activity	Binding activity	Activity	Binding activity	Activity	Binding activity
1	50% glycerol	3666	674	3689	593	101	88
2	10% Lys	2915	430	2221	345	76	80
3	10% Lys + 2% Ala	2894	412	2456	405	85	98
4	10% Lys + 2% Gly	2873	426	2475	389	86	91
5	10% Gly-Gly	2766	469	2527	419	91	89
6	10% Gly-Gly + 2% Ala	2722	461	2589	425	95	92
7	10% Gly-Gly + 2% Gly	2705	447	2572	428	95	96

(1) Additives in Nos. 3 to 7 were dissolved in a 20% glycerol solution.
(2) The initial values and values measured after the plasmin solution was allowed to stand at 37°C for 2 days are each indicated by the unit of Δ Abs/min.
(3) "Activity" means a titer to hydrolyze a plasmin synthetic substrate (S-2251).
(4) "Binding activity" means the binding ability of plasmin with α 2PI and is a value obtained by subtracting, from the activity of plasmin, residual plasmin activity at the time when the normal plasma specimen was reacted.
(5) "Ratio" means a ratio of the activity or binding activity measured after the plasmin solution was allowed to stand at 37°C for 2 days supposing that the activity or binding activity at the initial stage is 100%, respectively.
(6) Gly-Gly: glycyl glycine, Ala: alanine, Gly: glycine and Lys: lysine.

As is apparent from Table 2, it has been found that the plasmin solution according to the present invention was maintained stably without lowering the plasmin activity and α 2PI binding activity.

The plasmin solution No. 3 containing 10% lysine and 2% alanine had a viscosity of 1.40 cp when measured at 25°C by using VISCOMATE (manufactured by Yamaichi Denki Kogyo), while the plasmin solution No. 3 in Table 2 containing 10% lysine and 20% glycerol had a viscosity of 2.47 cp. On the other hand, the plasmin solution containing 50% glycerol had a viscosity of 6.98 cp. Thus, the plasmin solution according to the present invention has low viscosity so that it can be analyzed by an automatic analyzer.

Example 3

As in Example 1, various solutions having the compositions as shown in Table 3 were prepared and plasmin activity and binding activity of plasmin with α 2PI were measured at the time when the solutions were prepared and after they were stored at 37°C for 25 days. Incidentally, the physiological saline and normal plasma specimen were used each in an amount of 5 μ l. The results are shown in Table 3.

Table 3

No.	Additive	Initial value		Measured after 25 days at 37 °C		Ratio (%)	
		Activity	Binding activity	Activity	Binding activity	Activity	Binding activity
1	50% glycerol	3115	976	2917	704	94	72
2	Gly-Gly, Glycerol, L, A, G	2233	475	2240	515	100	108
3	Gly-Gly, Glycerol, L, A	2206	463	2109	478	96	103
4	Gly-Gly, Glycerol, L, G	2221	461	2168	457	98	99
5	Gly-Gly, Glycerol, A, G	2297	536	2228	538	97	101
6	Gly-Gly, Glycerol, L	2166	457	2050	468	95	102
7	Gly-Gly, Glycerol, A	2261	518	2185	523	97	101
8	Gly-Gly, Glycerol, G	2236	510	2150	517	96	101
9	Gly-Gly, Glycerol	2288	557	2100	541	92	97

(1) The initial values and values measured after the plasmin solution was allowed to stand at 37°C for 25 days are each indicated by the unit of Δ Abs/min.

(2) "Activity" means a titer to hydrolyze a plasmin synthetic substrate (S-2251).

(3) "Binding activity" means the binding ability of plasmin with α 2PI and is a value obtained by subtracting, from the activity of plasmin, residual plasmin activity at the time when the normal plasma specimen was reacted.

(4) "Ratio" means a ratio of the activity or binding activity measured after the plasmin solution was allowed to stand at 37°C for 25 days supposing that the activity or binding activity at the initial stage is 100%, respectively.

(5) Gly-Gly: 10% glycyL glycine, L: 2% lysine, A: 2% alanine, G: 2% glycine and Glycerol: 10% glycerol.

As is apparent from the results of Table 3, it has been found that the plasmin solution of the present invention was maintained stably without deteriorations in the plasmin activity and α 2PI binding activity, and that the plasmin solution had a low viscosity.

Example 4

As in Example 1, plasmin activity and plasmin α 2PI binding activity of various plasmin solutions having the compo-

sitions as shown in Table 4 were measured at the time when they were prepared and after they were stored at 37°C for 9 days. Incidentally, the physiological saline and the normal plasma specimen were used each in an amount of 5 µl. The results are shown in Table 4.

Table 4

No.	Additive	Initial value		Measured after 9 days at 37 °C		Ratio (%)	
		Activity	Binding activity	Activity	Binding activity	Activity	Binding activity
1	10% Gly-Gly	2675	607	2048	492	77	81
2	10% Gly-Gly + 10% Glycerol	2842	572	2685	580	95	101
3	10% Gly-Gly + 10% EtGlycol	2921	582	2645	561	91	96

(1) The initial values and values measured after the plasmin solution was allowed to stand at 37°C for 9 days are each indicated by the unit of ΔAbs/min.
 (2) "Activity" means a titer to hydrolyze a plasmin synthetic substrate (S-2251).
 (3) "Binding activity" means the binding ability of plasmin with α2PI and is a value obtained by subtracting, from the activity of plasmin, residual plasmin activity at the time when the normal plasma specimen was reacted.
 (4) "Ratio" means a ratio of the activity or binding activity measured after the plasmin solution was allowed to stand at 37°C for 9 days supposing that the activity or binding activity at the initial stage is 100%, respectively.
 (5) Gly-Gly: glycyl glycine, Glycerol: 10% glycerol and EtGlycol: ethylene glycol.

As is apparent from the results of Table 4, it has been found that the plasmin solution of the present invention containing glycyl glycine was maintained stably without deteriorations in the plasmin activity and binding activity of α2PI.

The plasmin solution No. 2 containing 10% glycyl glycine and 10% glycerol had a viscosity of 1.89 cp as measured at 25°C by VISCOMATE (manufactured by Yamaichi Denki Kogyosha). The plasmin solution containing 50% glycerol, on the other hand, had a viscosity of 6.98 cp at 25°C. The plasmin solution according to the present invention has thus a low viscosity and can therefore be provided for use in the measurement using an automatic analyzer.

Example 5

As in Example 1, the activity of α2PI of each of the specimen (n=2) having a concentration of 0 to 200% and α2PI specimen (n=10) having a concentration of 50% or 100% was measured using a plasmin solution of 10% glycyl glycine containing 10% glycerol, whereby the calibration curve exactness and its reproducibility were studied. Upon the study of the calibration curve exactness, the specimen was used in an amount of 10 µl because the stock solution of the normal plasma specimen was set to have an α2PI concentration of 200%. Upon the study of the reproducibility, the specimen was used in an amount of 5 µl because the stock solution of the normal plasma specimen was set to have an α2PI concentration of 100%. The results are shown in FIG. 1 and Table 5.

Table 5

Concentration (%)											Average	SD	CV
50%	52	55	54	55	57	54	57	57	60	58	56	2.33	4.17
100%	106	105	105	106	107	99	104	102	105	107	105	2.46	2.35

It has been found that from the results of FIG. 1, the plasmin solution had a good calibration curve property and from the results of Table 6, it has good reproducibility.

Industrial Applicability

The plasmin solution according to the present invention can be maintained stably without deteriorations in the plasmin activity and binding activity with α2PI even after long term storage and is therefore useful as a reagent for the quan-

titative measurement of α 2PI. Moreover, it can be stored stably in the form of a solution so that it can be used as is at the time of measurement, which makes it possible to carry out measurement conveniently and promptly with excellent economy and operability. Furthermore, it has low viscosity so that it can be suitably used in the measurement by an automatic analyzer.

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Claims

1. A plasmin solution comprising:

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(A) plasmin, and
(B) the following component (B-1), (B-2) or (B-3) :

(B-1) an oligopeptide comprising at least two amino acids bonded each other, said two amino acids being selected from lysine, arginine, glycine, alanine, aspartic acid and methionine;

15

(B-2) at least two amino acids selected from lysine, arginine, glycine, alanine, aspartic acid and methionine; or

(B-3) an amino acid selected from lysine, arginine, glycine, alanine, aspartic acid and methionine and a polyhydric alcohol.

20

2. A plasmin solution according to claim 1, which comprises (B-1) or (B-2) as the component (B) and further comprises a polyhydric alcohol.

3. A plasmin solution according to claim 1, which comprises (B-1) as the component (B) and further comprises at least one amino acid selected from lysine, arginine, glycine, alanine, aspartic acid and methionine.

25

4. A plasmin solution according to claim 3, further comprising a polyhydric alcohol.

5. A process for stabilizing plasmin, which comprises adding, to a plasmin solution, as an additive:

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(B-1) an oligopeptide comprising at least two amino acids bonded each other, said two amino acids being selected from lysine, arginine, glycine, alanine, aspartic acid and methionine;

(B-2) at least two amino acids selected from lysine, arginine, glycine, alanine, aspartic acid and methionine; or

(B-3) an amino acid selected from lysine, arginine, glycine, alanine, aspartic acid and methionine and a polyhydric alcohol.

35

6. A process according to claim 5, which comprises adding (B-1) or (B-2) as the additive and further comprising adding a polyhydric alcohol.

40

7. A process according to claim 5, which comprises adding (B-1) as the additive and further comprises adding at least one amino acid selected from lysine, arginine, glycine, alanine, aspartic acid and methionine

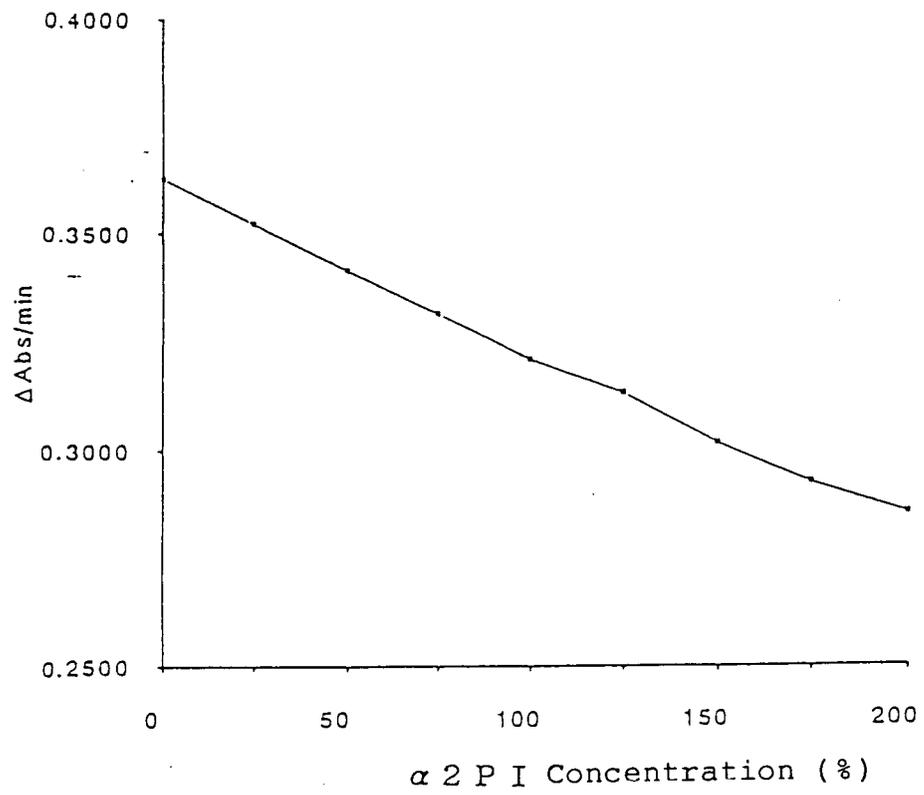
8. A process according to claim 7, which further comprises adding a polyhydric alcohol.

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Fig. 1



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP96/01738

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl ⁶ C12N9/96; C12Q1/37, 1/56 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Int. Cl ⁶ C12N9/96; C12Q1/37, 1/56 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) JICST File on Science and Technology		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, 3-66292, B2 (BEHRINGWERKE AG.), October 16, 1991 (16. 10. 91) & EP, B, 65256 & US, A, 442213	1-2, 5-6
A	JP, 62-36502, B2 (The Green Cross Corp.), August 7, 1987 (07. 08. 87) & GB, B, 2068002	1 - 8
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Date of the actual completion of the international search July 15, 1996 (15. 07. 96)	Date of mailing of the international search report July 30, 1996 (30. 07. 96)	
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